A vasopressin/oxytocin-related conopeptide with γ-carboxyglutamate at position 8

Carolina MÖLLER and Frank MARÍ[1]

Department of Chemistry and Biochemistry, and Center of Excellence in Biomedical and Marine Biotechnology, Florida Atlantic University, Boca Raton, FL 33431, U.S.A.

Vasopressins and oxytocins are homologous, ubiquitous and multifunctional peptides present in animals. Conopressins are vasopressin/oxytocin-related peptides that have been found in the venom of cone snails, a genus of marine predatory molluscs that envenom their prey with a complex mixture of neuroactive peptides. In the present paper, we report the purification and characterization of a unique conopressin isolated from the venom of *Conus villepinii*, a vermivorous cone snail species from the western Atlantic Ocean. This novel peptide, designated γ-conopressin-vil, has the sequence CLIQDCPγG* (γ is γ-carboxyglutamate and * is C-terminal amidation). The unique feature of this vasopressin/oxytocin-like peptide is that the eighth residue is γ-carboxyglutamate instead of a neutral or basic residue; therefore it could not be directly classified into either the vasopressin or the oxytocin peptide families. Nano-NMR spectroscopy of the peptide isolated directly from the cone snails revealed that the native γ-conopressin-vil undergoes structural changes in the presence of calcium. This suggests that the peptide binds calcium, and the calcium-binding process is mediated by the γ-carboxyglutamate residue. However, the negatively charged residues in the sequence of γ-conopressin-vil may mediate calcium binding by a novel mechanism not observed in other peptides of this family.

Key words: γ-carboxyglutamate, conopressin, *Conus villepinii*, oxytocin, vasopressin, venom.

INTRODUCTION

Vasopressin and oxytocin are ubiquitous hormone peptides present in various animal species. All vasopressin/oxytocin-related peptides have a very similar primary structure. They are cyclic nonapeptides with one disulfide bond and a glycylamide C-terminal residue. The cyclic hexapeptide ring structure (20-membered ring) is defined by the disulfide bond between the cysteines at residues 1 and 6. The sequence is terminated by a highly flexible C-terminal three-residue tail that contains the critical eighth amino acid, as these peptides are structurally and functionally distinguished by the chemical nature of the amino acid residue at position eight. In the vasopressin peptide family, this residue is a basic amino acid, whereas in the oxytocin peptide family, it is a neutral amino acid [1]. Structurally, these hormones and their analogues are very flexible in aqueous solution; however, some of these hormones have been reported to adopt well-defined structures in mixtures of water and organic solvents [2,3]. Cations may affect the conformation of these hormones in a biologically relevant context, i.e. Ca^{2+} induced conformational changes in both the cyclic moiety and in the tail tripeptide following a two-step process [4,5].

In mammals, vasopressin and oxytocin peptides are multifunctional hormones that carry out important functions in osmoregulation and endocrine control. In the brain, vasopressin is released within distinct areas upon appropriate stimulation, including stressful challenges [6]. Oxytocin regulates a large number of reproduction-related processes in all species and it is essential for milk delivery during lactation [7]. Oxytocin also has a role as a neurotransmitter mediating reproduction-related processes such as maternal behaviour, sexual receptivity and partnership bonding [8]. However, vasopressin/oxytocin-related peptides are not restricted to vertebrates; they have been isolated from several invertebrate species, such as cone snails [9,10], octopuses [11,12], leeches [13], insects [14] and earthworms [15]. The presence of vasopressin/oxytocin-related peptides in invertebrates is thought to be a duplication of the ancestral gene which occurred before the divergence of vertebrates and invertebrates over 600 million years ago [16].

Vasopressin/oxytocin-related peptides carry out their function by binding to extracellular domains of GPCRs (G-protein-coupled receptors), which in turn propagate the signal intracellularly. Although direct structural details of these receptors and the way they bind to the hormones are still lacking, extensive mutagenesis and binding studies of analogues have provided an understanding of the key factors that govern the relationship between structure and activity of theses hormones and their receptors, i.e. it has been determined that the side chain of the eighth residue of vasopressin interacts with a non-conserved receptor residue located in the first extracellular loop. This interaction is completely responsible for the selectivity of the ligand–receptor interaction [17].

Vasopressin/oxytocin-related peptides found in the venom of cone snails are called conopressins [9,10]. Conopressins were originally discovered by bioassay-driven fractionation of cone snail venom, where, upon intracerebroventricular injection in mice, an intense 'scratching' effect was induced [9]. Cone snails are predatory marine molluscs that use venom to capture prey. The venom is a complex mixture of neuroactive peptides (conopeptides). Most conopeptides specifically target voltage-gated ion channels and ligand-gated receptors [18]. Conopeptides

Abbreviations used: 1D, one-dimensional; 2D, two-dimensional; DSV, deep submersible vehicle; DTT, dithiothreitol; FID, free induction decay; Gla, γ-carboxyglutamate; GPCR, G-protein-coupled receptor; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; NOE, nuclear Overhauser effect; PFG, pulse-field gradient; PTH, phenylthiohydantoin; RF, radio frequency; ROE, rotating-frame Overhauser effect; ROESY, rotating-frame Overhauser enhancement spectroscopy; RP, reverse-phase; SE, size-exclusion; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TSP, 3-(trimethylsilyl)-[$^2H_4$]propionic acid sodium salt; *w*, WET; *wg*, WATERGATE.

[1] To whom correspondence should be addressed (email mari@fau.edu).

The sequence of γ-conopressin-vil has been deposited in the UniProt Knowledgebase under accession number P85141.

Figure 1 ***Conus villepinii*** **is a widespread western Atlantic Ocean deep-water cone snail species**

Its habitat expands from the Florida coast to Brazil (photograph by William P. Cargile).

posses a high degree of post-translationally modified residues (usually combinations of them), such as γ-carboxyglutamate, γ-hydroxyproline, δ-hydroxylysine, D-γ-hydroxyvaline, D-tryptophan, D-leucine, D-phenylalanine [19], D-isoleucine, D-methionine [20], bromotryptophan, pyroglutamate, glycosylated serine/threonine, sulfated tyrosine and cystine bridges in multiple patterns [21,22]. These modifications confer on conopeptides a unique stability and specificity towards neuronal targets [23], enabling cone snails to capture prey.

We isolated a vasopressin/oxytocin-like peptide (γ-conopressin-vil) from the venom of *Conus villepinii*, a worm-hunting cone snail species of the western Atlantic Ocean that inhabits deep waters (Figure 1). The unique feature of this peptide is that the eighth amino acid is γ-carboxyglutamate (Gla) instead of a neutral or basic residue. To our knowledge, no other vasopressin/oxytocin-like peptide with an added post-translational modification (besides the cystine bridge and the amidated C-terminus) has been reported. The γ-carboxylation of glutamate residues is carried out by the vitamin K-dependent enzyme γ-glutamyl carboxylase, only functionally expressed in invertebrates by cone snails [24] and by the sea slug *Aplysia californica* [25]. Using 2D (two-dimensional) NMR spectroscopy, we found that γ-conopressin-vil undergoes structural changes upon calcium binding in a process that is mediated by the γ-carboxyglutamate residue.

MATERIALS AND METHODS

Specimen collection

Specimens of *C. villepinii* (30–80 mm long) were collected off the Florida Keys (Marathon Key, FL, U.S.A.) using a Capetown dredge deployed from oceanographic research vessels, R/V Suncoaster and R/V Bellows, at depths ranging from 100 to 200 m. Additional snails were collected using the Johnson-Sea Link DSV (deep submersible vehicle), operated from the R/V Seward Johnson, and working at a depth of 200 m at the same location as indicated above. Cone snails were collected using a suction device attached to a robotic arm of the DSV. All snails were kept in aquaria before transportation to the lab, where they were dissected and immediately frozen at $-80\,^{\circ}C$.

Crude venom extraction

Venom ducts dissected from 63 specimens of *C. villepinii* were homogenized in 0.1 % TFA (trifluoroacetic acid) at 4 °C. Whole extracts were centrifuged at 10000 ***g*** for 20 min at 4 °C. The resulting pellets were washed three times with 0.1 % TFA and re-centrifuged under similar conditions. The supernatants containing the soluble peptides were pooled, freeze-dried and stored at $-80\,^{\circ}C$ until further use.

Peptide purification

Crude venom was initially fractionated by SE (size-exclusion) HPLC on a Pharmacia Superdex-30 column (2.5 cm × 100 cm) equilibrated and eluted with 0.1 M NH_4HCO_3 using a flow rate of 1.5 ml/min. Chromatographic fractions were monitored at wavelengths of 220, 250 and 280 nm. Additional purification of peptide-containing peaks was achieved by RP (reverse-phase) HPLC on a C_{18} semi-preparative column [Vydac 218TP510, 10 mm × 250 mm; 5 μm particle diameter; 300 Å (1 Å = 0.1 nm) pore size] equipped with a C_{18} guard column (Upchurch Scientific, AC-43 4.6 mm) at a flow rate of 3.5 ml/min. Further peptide purification was carried out by re-chromatographing fractions on an analytical C_{18} column (Vydac 238TP54, 4.6 mm × 250 mm; 5 μm particle diameter; 300 Å pore size) with a flow rate of 1 ml/min. For semi-preparative and analytical RP HPLC separation, the solvents were 0.1 % TFA (solvent A) and 0.1 % TFA in 60 % acetonitrile (solvent B). Peptides were eluted with an incremental linear gradient of 1 % solvent B/min. Absorbances were monitored at wavelengths of 220 and 280 nm. All HPLC fractions were collected manually, freeze-dried and kept at $-40\,^{\circ}C$ until further use.

Reduction and alkylation of cysteine residues

Reduction and alkylation of cystine groups were carried out as described previously [26] with slight modifications. An aliquot of each peptide (~ 1 pmol) was dried, redissolved in 0.1 M Tris/HCl (pH 6.2), 5 mM EDTA and 0.1 % sodium azide, and reduced with 6 mM DTT (dithiothreitol). Following a 30 min incubation at 60 °C, peptides were alkylated in a final volume of 15 μl with 20 mM iodoacetamide and 2 μl of NH_4OH (pH 10.5) at room temperature (25 °C) for 1 h in the dark. The reduced and alkylated peptides were purified using a ZipTip (C_{18}, size P10; Millipore).

Peptide sequencing

Alkylated peptides were adsorbed on to Biobrene-treated glass fibre filters, and amino acid sequences were determined by Edman degradation using an Applied Biosystems Procise model 491A sequencer. The concentration of the peptides was determined by using the calibrated intensities of the first five PTH (phenylthiohydantoin)–amino acid residues on samples that were not reduced and alkylated.

Molecular mass determination

Positive-ion MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS was carried out on an Applied Biosystems Voyager-DE STR spectrometer. Samples were dissolved in 0.1 % TFA and 50 % acetonitrile and applied on to a α-cyano-4-hydroxycinnamic acid matrix. Spectra were obtained in the linear and reflector modes using Calmix 1 and Calmix 2 (Applied Biosystems) as external calibration standards.

Calcium binding of γ-conopressin-vil

Freeze-dried peptide was dissolved in water to a final concentration of 0.7 μM. $CaCl_2$ was added to a final concentration of 6 mM and sample pH was adjusted to 6.2.

NMR spectroscopy

NMR spectra were acquired on a Varian Inova 500 MHz instrument equipped with PFG (pulse-field gradient), 3 × RF (radio frequency) channels and waveform generators. Nanomolar quantities (4 nmol) of the native conopeptide directly isolated from the venom were dissolved in either 40 μl of water with 10 % ^2H_2O (used for locking purposes) or 40 μl of 50 % [$^2H_3$]TFE (trifluoroethanol) in water with 4 nmol of TSP {3-(trimethylsilyl)-[$^2H_4$]propionic acid sodium salt} and placed in 1.7 mm NMR tubes (Wilmad WG-1364-1.7). The pH was adjusted using 0.01 M solutions of HCl and NaOH and a Thermo micro-pH probe. Spectra were obtained using a Varian *g*HCN (generation 5) high-performance 3 mm probe [pw90 (90° pulse width) = 3 μs], at the upper limit of the linear range of the RF amplifier) with a 1.7 mm capillary adaptor (Wilmad V-GFK-10/1.7). NMR experiments were conducted as described previously [26] at pH 5.8 and at different temperatures (0, 10, 15 and 25 °C) in order to achieve the best chemical-shift dispersion possible to aid the sequence-specific assignments. For 1D (one-dimensional) NMR experiments, the water signal was suppressed by using either double spin-echo [27] or pre-saturation. Peptide concentrations were evaluated by integrating the NMR signals of selected methyl groups and using the known concentration of TSP as an internal standard or the signal of selected methyl groups from peptides with known concentration as external standards. For 2D-TOCSY and 2D-NOESY experiments, water suppression was carried out using WATERGATE (*wg*) in combination with 3919 purge pulses with flipback, which was implemented in the TOCSY and NOESY pulse sequences. For 2D-ROESY (rotating-frame Overhauser enhancement spectroscopy) experiments, water suppression was carried out using WET (*w*) [28]. The *wg*TOCSY, *w*ROESY and *wg*NOESY experiments were used to obtain information on sequence-specific assignments and the secondary structure of the peptide. All 2D-NMR spectra were recorded in the phase sensitive mode using the States–Haberkorn method with a spectral width of 6000 Hz and 2000 data points. For the *wg*TOCSY experiment, 160 scans for each of the 96 FIDs (free induction decays) were acquired with relaxation delay of 1.7 s and a mixing time of 120 ms. The 2D *wg*NOESY and *w*ROESY spectra of the conopeptide were recorded using 256 scans for each of the 128 FIDs, acquired with a 1.7 s relaxation delay and a mixing time of 200 ms. All 2D-NMR data were processed using VNMR 6.1C (Varian NMR Instruments) on Sun Blade 150 workstations. FIDs were apodized with a shifted sine bell window function, linearly predicted to 1000 points in *t*1, and zero-filled to 2000 × 2000 data matrices. The data was baseline corrected in F2 by applying a polynomial function. Sequence-specific assignments of all proton resonances were carried out using standard biomolecular NMR procedures.

Nomenclature

Conopeptides isolated from *C. villepini* are to receive the three letter 'vil' designation [26]. Following the nomenclature used for conopressins, we decided to name the peptide γ-conopressin-vil because the eighth residue is a carboxyglutamate acid.

RESULTS

Peptide purification

Crude venom from *C. villepinii* (113 mg) was initially fractionated using SE HPLC on the Superdex 30 column (Figure 2A). The SE fractions identified by the arrows in Figure 2(A) were pooled and rechromatographed on a semi-preparative C_{18} column (Figure 2B), and the purification of a single component was achieved using an analytical C_{18} column (inset).

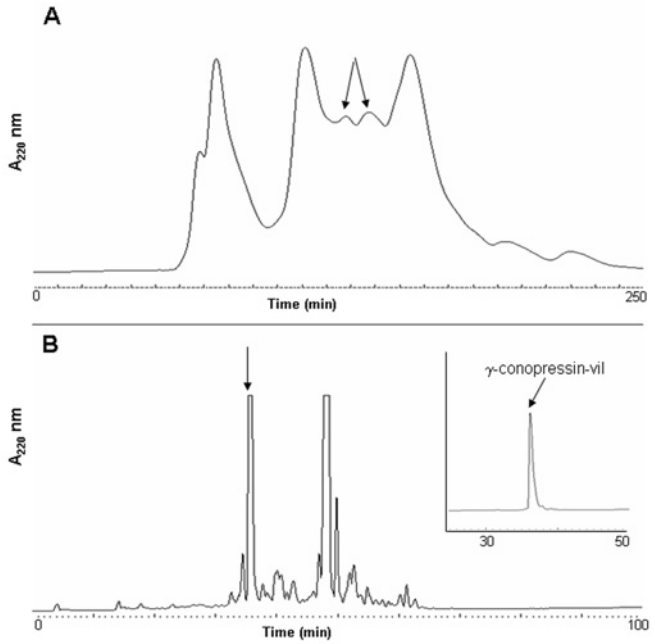

Figure 2 Purification of the conopeptide γ-conopressin-vil

(**A**) Separation by SE HPLC (Superdex 30). The column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 1.5 ml/min. Peptide γ-conopressin-vil was purified from the peaks marked with the arrows. (**B**) Fractions were pooled and applied to a Vydac C_{18} semi-preparative column and eluted with a linear gradient of 1 % solvent B increase per min for 100 min at a flow rate of 3.5 ml/min. The peak highlighted by the arrow was purified further on an analytical Vydac C_{18} column using the same gradient of 100 min at 1 ml/min (inset). For both semi-preparative and analytical RP HPLC, the eluting solutions were (**A**) 0.1 % TFA and (**B**) 60 % acetonitrile with 0.1 % TFA.

MS of purified peptide

MS was carried out by MALDI–TOF MS in the reflector mode (M/ΔM resolution ∼10000). The monoisotopic molecular ion obtained for the peptide was 996.45 Da, corresponding to the molecular ion of the peptide plus a sodium ion (Figure 3). The presence of one γ-carboxyglutamate residue in the peptide is distinguished in the spectrum by the losses of 44 Da owing to decarboxylation.

Reduction and alkylation

The purified peptide was subjected to reduction with DTT and alkylation with iodoacetamide. Reduction and alkylation of cysteine residues with iodoacetamide resulted in a mass increase of 116.05 Da as compared with the native compound, which indicated the presence of two cysteine residues. Also, the molecular ion of the reduced/alkylated peptide was accompanied by loss of 44 Da owing to decarboxylation of the γ-carboxyglutamate residue (Figure 3).

Peptide sequence determination

The reduced/alkylated peptide was sequenced to completion by Edman degradation. The sequence of γ-conopressin-vil is shown in Table 1. The experimental molecular mass (see above) was in agreement with the calculated theoretical monoisotopic value.

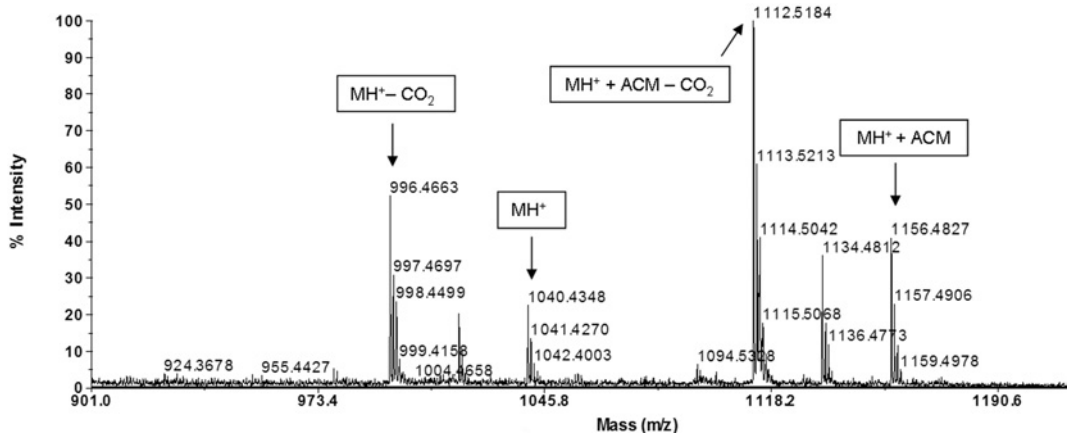

Figure 3 MALDI–TOF mass spectra (reflector mode) of the partially reduced and alkylated peptide γ-conopressin-vil

The molecular ion at 1040.43 shows the loss of 44 Da consistent with the presence of one γ-carboxyglutamate residue (996.47). The difference of 116.05 Da between the native (MH^+) and reduced and alkylated peptide [MH^+ + acetamide (ACM)] is indicative of the presence of two cysteine residues.

Table 1 Vasopressin/oxytocin-related peptides

Data taken from [9–11,14,15,33,49]. γ, γ-carboxyglutamate; *, C-terminal amidation.

Receptor	Peptide	Sequence	Organism	Net charge at pH 7
Unknown	γ-Conopressin-vil	CLIQDCPγG*	*C. villepinii*	−2
Vasopressin	Lys-conopressin-G	CFIRNCPKG*	*Lymnaea stagnalis*, *C. geographus*, *Conus imperialis*, *Aplysia kurodal*	+3
	Arg-conopressin-S	CIIRNCPRG*	*Conus striatus*	+3
	Arg-conopressin-T	CYIQNCLRV*	*Conus tulipa*	+2
	Diuretic hormone	CLITNCPRG*	*Locusta migratoria*	+2
	Vasotocin	CYIQNCPRG*	Non-mammalian vertebrates	+2
	Arg-vasopressin	CYFQNCPRG*	Mammals	+2
	Lys-vasopressin	CYFQNCPKG*	Mammals	+2
	Phenypressin	CFFQNCPRG*	Mammals	+2
Oxytocin	Cephalotocin	CYFRNCPIG*	*Octopus vulgaris*	+2
	Annetocin	CFVRNCPTG*	*Eisenia foetida*	+2
	Aspargtocin	CYINNCPLG*	Cartilaginous fish (sharks)	+1
	Asvatocin	CYINNCPVG*	Cartilaginous fish (sharks)	+1
	Phasvatocin	CYFNNCPVG*	Cartilaginous fish (sharks)	+1
	Valitocin	CYIQNCPVG*	Cartilaginous fish (sharks)	+1
	Glumitocin	CYISNCPQG*	Cartilaginous fish (rays)	+1
	Isotocin	CYISNCPIG*	Bony fish	+1
	Mesotocin	CYIQNCPIG*	Mammals, birds, reptiles, amphibia, lungfish	+1
	Oxytocin	CYIQNCPLG*	Mammals	+1

The difference of 1 Da between the monoisotopic molecular mass obtained by MALDI–TOF MS and the calculated mass suggests that γ-conopressin-vil is amidated on the C-terminal glycine residue, in agreement with the other vasopressin/oxytocin-related peptides. The peptide contained nine residues with two cysteine residues separated by four residues, and the sequence resembles the vasopressin/oxytocin family. However, γ-conopressin-vil has a γ-carboxyglutamate residue at position 8. The γ-carboxyglutamate residue was determined by the presence of a weak increase in the yield of PTH–Glu peak, typical for γ-carboxyglutamate residues [29].

NMR spectroscopy

We were able to obtain NMR spectra (1D and 2D) of nanomolar quantities (nano-NMR) of the γ-conopressin-vil isolated directly from the venom of the cone snails. Figure 4 shows portions of the *wg*TOCSY and *w*ROESY spectra of γ-conopressin-vil at 0 °C in water. As in previous NMR studies of vasopressin/oxytocin-related peptides, γ-conopressin-vil has a discrete number of ROE (rotating-frame Overhauser effect)/NOE (nuclear Overhauser effect) (< 50) cross-correlations. The number of dipolar connectivities observed in the ROESY spectra is the same as in the NOESY spectra. Spectra recorded in 50 % [$^2H_3$]TFE in water also resulted in a similar number of ROE/NOE cross-correlations. Spectra at higher temperatures (10, 15 and 25 °C) resulted in lower numbers of ROE/NOE cross-correlations (results not shown). γ-Conopressin-vil is very flexible in solution, regardless of the temperature or medium used. Sequence-specific assignments for all protons in γ-conopressin-vil were achieved by analysis of *wg*TOCSY and *w*ROESY spectra in water (Figure 4). The presence of the γ-carboxyglutamate residue was confirmed by its characteristic spin system chemical shifts, which include a γH signal at 3.38 p.p.m.

Calcium binding of γ-conopressin-vil

To determine the structural changes of γ-conopressin-vil in the presence of calcium, we collected ^{1}H 2D-NMR NOESY and

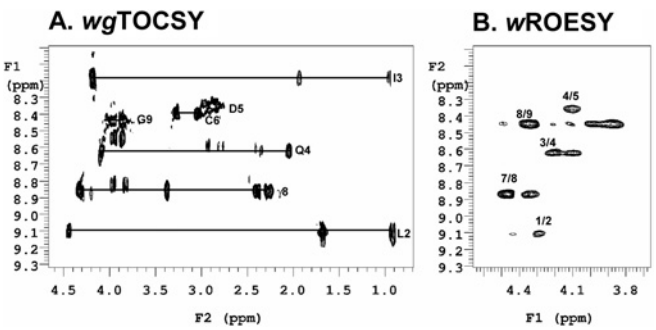

Figure 4 TOCSY and ROESY spectra of γ-conopressin-vil

(**A**) 2D ^{1}H-NMR *wg*TOCSY of 4 nmol of γ-conopressin-vil at 0 °C in water. (**B**) 2D ^{1}H-NMR *w*ROESY of 4 nmol of γ-conopressin-vil at 0 °C in water; the numbering (αH/HN) indicates cross-peaks between αHs and HNs. The proton chemical shifts for each residue were assigned according to standard procedures [50].

TOCSY spectra in water containing 10 % ^2H_2O and in 50 % [$^2H_3$]TFE in water. Proton chemical-shift assignments were obtained for both the free and the Ca^{2+}-bound forms of the peptide. The addition of Ca^{2+} causes changes in the resonances of some of the amino acids (Figure 5).

The ROE cross-peaks between the αH of one residue (i) and HN of the subsequent residue ($i + 1$) were used for the sequential assignments along with cross-peaks between amide protons. We found very few connectivities in the HN region (results not shown), which indicates that the structure of the peptide is not helical in either water or TFE/water mixtures, regardless of the presence of Ca^{2+} ions. This is in agreement with previous NMR studies [30] where the oxytocin structure was shown to contain two β-turns in the ring and a flexible tail. Resonance assignments in the HN–αH/side-chain region revealed a total of $\sim$25 inter-proton correlations for the free and Ca^{2+}-bound peptide.

The changes in the proton chemical shifts, $\Delta\delta_{Ca}$, between the calcium-free γ-conopressin-vil and in presence of calcium in water containing 10 % ^2H_2O and 50 % TFE in water solutions are shown in Figure 5. The results in Figure 5 suggest that the addition of Ca^{2+} alters the proton resonances in different ways. The HN protons of Gln^4, Gla^8 and Gly^9 show major Ca^{2+}-induced changes in $\Delta\delta_{Ca}$, which is an indication that these residues are involved in metal binding. Upon calcium addition, the HN protons for the amino acids Gln^4 and Gly^9 were more deshielded, whereas the HN of Gla^8 was significantly shielded. Important $\Delta\delta_{Ca}$ changes of the αHs were observed for Cys^1, Asp^5 and Gla^8, as these residues were more deshielded in the presence of the metal ion. The αHs of Ile^3, Cys^6 and Gly^9 were shielded by the addition of Ca^{2+}. The presence of Ca^{2+} also shielded the β-protons of Cys^1 and the γ-protons of Gln^4 and Gla^8 (results not shown). The latter is particularly important, since the two carboxy groups are bonded to the γ-carbon of the γ-carboxyglutamate residue, and the shift of its corresponding γH is expected to be sensitive to calcium binding [31].

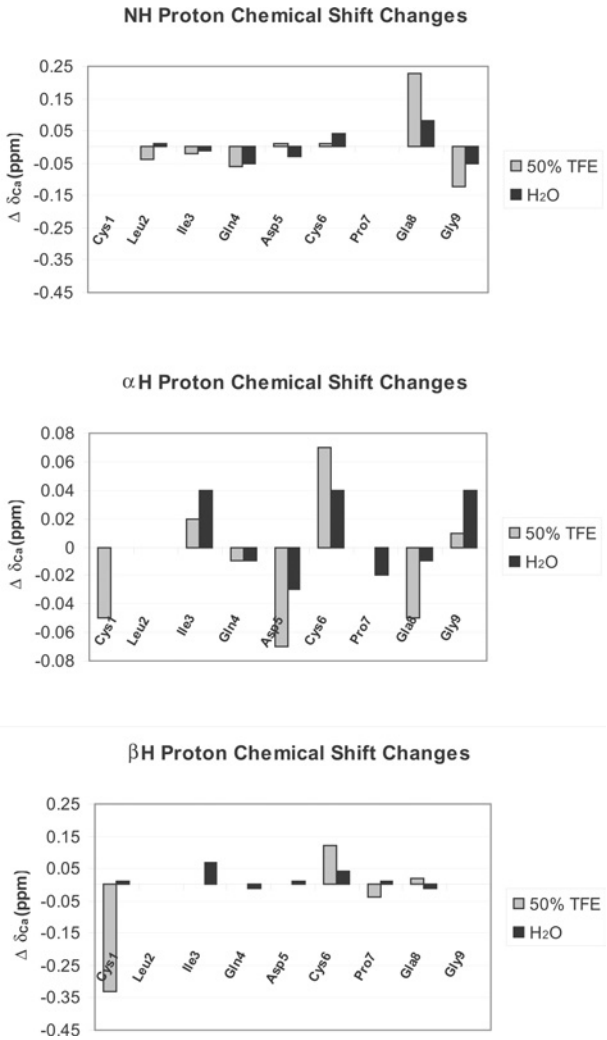

Figure 5 Proton chemical-shift changes ($\Delta\delta_{Ca}$, in p.p.m.) in the γ-conopressin-vil peptide upon the addition of Ca^{2+} in water containing 10 % ^2H_2O and 50 % [$^2H_3$]TFE in water at 0 °C

(**A**) HN proton chemical-shift changes. (**B**) αH proton chemical-shift changes. (**C**) βH proton chemical-shift changes.

DISCUSSION

The sequence of γ-conopressin-vil diverges significantly from the other known peptides of the oxytocin/vasopressin family, particularly by the presence of a post-translationally modified amino acid γ-carboxyglutamate at the critical eighth position (Table 1). This is in contrast with previous findings that suggest that the chemical nature of the amino acid residue at the eighth position is not as important in invertebrates [32]. Furthermore, the conopressins characterized so far are structurally related to vasopressin, as they have lysine or arginine at position 8; however, they elicit functional characteristics that are more typical for oxytocin. Since γ-conopressin-vil has a γ-carboxyglutamate residue in the eighth position, it could not be classified *a priori* as either a vasopressin or an oxytocin. γ-Conopressin-vil also lacks the conserved asparagine residue at the fifth position; instead, an aspartate residue occupies this position, providing an additional negative charge that may be involved in calcium binding.

To date, only three conopressins have been characterized: conopressin-G [33], conopressin-S [10] and conopressin-T [33]. The ubiquitous finding of conopressins in many organisms, particularly in molluscs, is an indication of their important role in their endocrine system; however, the function of the conopressins present in the venom is still unknown. Conopressins may be secreted by the neuronal tissue of the venom duct and can also play a role as toxins [9]. Other modified endogenous peptides, such as the conomaps [34] and conorfamides [35], have been found in *Conus* venom, and it has been suggested that modifications of these peptides improves venom potency [34]. In the

case of γ-conopressin-vil, the γ-carboxyglutamate modification appears to have a special connotation, as this is a very exacting modification for conopeptides. Carboxylation of glutamate is specific and is only observed in the venom of certain species and in certain peptides of a family of conopeptides; i.e. out of the six α-conotoxins found in the venom of *Conus geographus*, GID is the only one that is carboxylated [36]. Nevertheless, the γ-carboxyglutamate modification in conopeptides is multifunctional, as the presence of multiple γ-carboxyglutamate residues structurally defines the conantokins, their calcium-binding properties and NMDA (*N*-methyl-D-aspartate) receptor targeting [37]. Also, the γ-carboxyglutamate residues in Gla-contryphan-M are essential for blockage on the L-type calcium channel [38].

The γ-conopressin-vil peptide has Asp^5 and Gla^8, as opposed to Asn^5 and a neutral or negative eighth residue typical for the vasopressin/oxytocin family. It has a net charge of −2, which is unique when compared with the +2/+3 and +1 for vasopressins and oxytocins respectively (see Table 1). These charge differences significantly affect the electrostatic surfaces of these peptides, which in turn will affect the binding modes to their targets. NMR analysis of the peptide in the presence of calcium shows that the ion induces conformational changes in γ-conopressin-vil. This has also been observed in other peptide hormones that bind Ca^{2+} or Mg^{2+} with enhanced affinity to the receptor, forming a ternary complex between the hormone, receptor and the cation [39]. In the case of γ-conopressin-vil, γ-carboxyglutamate confers to this conopressin a unique calcium-binding property, which suggests a new receptor-binding mechanism for this hormone family.

γ-Conopressin-vil exhibits the same structural flexibility of other vasopressin/oxytocin-related peptides. This flexibility was also found to be independent of the medium used, either water or 50% TFE solutions. TFE provides a less polar medium than water, as it lowers the dielectric effect of the solution, providing a cell/membrane-like environment [40], which could be biologically relevant for certain peptides. However, in this particular case, 50% TFE solutions have little structural effect on γ-conopressin-vil. Ca^{2+} binding to γ-conopressin-vil has a slight effect on the conformational flexibility of the peptide. This flexibility is in part due to a *cis–trans* conformational equilibrium across the cysteine[6]–proline peptide bond [41]. The rate of *cis–trans* isomerism in the vasopressin/oxytocin-related peptides is unusually accelerated as a result of conformational constraints imposed by the disulfide bond [42]. This is also the case for γ-conopressin-vil, as a second set of minor signals was always present in our NMR spectra (1D and 2D), regardless of the medium used or the presence of Ca^{2+}. The pattern of these signals resembles the one observed for oxytocin [41].

The multifunctional role of vasopressin/ocytoxin peptides is intimately related to their ability to bind multiple targets. Proper folding and trafficking of vasopressin/oxytocin prohormones is controlled by the carrier protein neurophysin [43], such as conophysin-R, isolated from *Conus radiatus* venom [44]. The structure of oxytocin bound to neurophysin indicates that Cys^1 and Tyr^2 are the critical residues for binding to the carrier [45]. Structural details of vasopressin/oxytocin hormones bound to their GPCRs are not available yet; however, a crystal structure has been determined for the complex between vasopressin and trypsin, which reveals that vasopressin fulfils all of the important interactions for an inhibitor [46]. The trypsin-binding site is located at the C-terminus of the hormone, whereas the neurophysin-binding site is at the N-terminus of vasopressin. Vasopressin inhibition of trypsin implies that the hormone may have unexplored functions. The multifunctionality of the vasopressin/oxytocin family might be further extended by post-translational modifications such as carboxylation, as observed in γ-conopressin-vil. This modification has enabled the peptide to bind calcium, perhaps adding further biological connotations to these peptides. The different functions of the oxytocin and vasopressin offer a number of therapeutic possibilities via modulation of their receptor activities, such as the treatment of preterm labour, diabetes insipidus, hypertension, dysmenorrhoea, and use as an antidiuretic [47,48]. Finding and elucidating the role of new vasopressin/oxytocin-related peptides such as γ-conopressin-vil could expand the development of related novel therapeutic agents.

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